Isolation and Genetic Mapping of a Non-Lethal Rice (*Oryza sativa* L.) *low phytic acid 1* Mutation

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ABSTRACT

Phytic acid (*myo*-inositol 1,2,3,4,5,6 hexa*kis*phosphate) is the most abundant form of phosphorus (P) in seeds and is virtually indigestible by humans or non-ruminant livestock. It was hypothesized that one class of maize (*Zea mays* L.) and barley (*Hordeum vulgare* L.) *low phytic acid* mutations, designated *lpa1*, interrupt *myo*-inositol supply during seed development and may be mutations of the *myo*-inositol 1-phosphate synthase (MIPS) gene. This study describes the isolation, inheritance, and genetic mapping of the first rice *lpa1* mutation and reexamines the MIPS/*lpa1* candidate gene hypothesis in rice. Grain from 3632 rice M2 lines, derived from gamma-irradiated seed, was screened for the *lpa* phenotype. Two mutations, one lethal and one non-lethal, were identified. The non-lethal mutation is phenotypically similar to maize and barley *lpa1* mutants and was designated rice *lpa1-1*. Homozygosity for rice *lpa1-1* reduces the phytic acid portion of seed P from 71 to 39% and increases the inorganic portion of seed P from 5 to 32%, with little effect on total seed P. This rice *lpa1* mutation was mapped to a 2.2-cM interval on chromosome 2L. A single-copy rice MIPS gene was mapped to a locus on rice chromosome 3 that is orthologous to MIPS loci on maize chromosome 1S (near maize *lpa1*) and barley chromosome 4H. Unlike maize *lpa1*, the rice and barley *lpa1* mutations loci are clearly distinguishable from this canonical MIPS gene. No relationship can be inferred between the maize, barley, and rice *lpa1* loci. Although this canonical MIPS gene may be an appropriate target for controlling seed phytic acid synthesis, modifications of other genes (e.g., maize *lpa2*, barley *lpa1*, barley *lpa2*, and rice *lpa1*) may also be useful in reducing grain phytic acid and improving the nutritional value of cereal grains and/or milling by-products.

Myo-inositol (Ins) 1,2,3,4,5,6-hexa*kis*phosphate (Ins P_6) is ubiquitous in eukaryotic cells, where it is typically the most abundant inositol phosphate (Ins P) (Stephens et al., 1993). This compound was first observed as an abundant P-containing compound in seeds, and thus referred to as "phytic acid" in the plant sciences and agronomic literature (Cosgrove, 1980). The applied interest in seed phytic acid concerns its role in livestock and human nutrition as well as P management in agricultural production. In terms of cell biology, many studies in plant and animal systems indicate that Ins P_6/phytic acid is a major pool in both P and Ins P metabolism (Stephens et al., 1993; Raboy, 1997). When plants are grown under nominal or non-extreme conditions, phytic acid P typically represent from 65 to 85% of seed total P and greater than 95% of free Ins polyphosphates (Raboy, 1997). Trace levels (<5% of total Ins P) of "lower" Ins polyphosphates (Ins bis-, tris-, tetra*kis*-, and penta*kis*phosphates) are also often observed in mature, wild-type seeds. Normally, inorganic P (P_i) typically represents about 5% (±3%) of seed total P and all other forms of organic P (DNA, RNA, free nucleotides, phospholipids, sugar phosphates, etc.), referred to here as *cellular P*, represent about 10 to 20% of seed total P. Substantial variation in seed total P of a given line or genotype can result from environmental or genotypic factors that alter the supply of P to the developing seed. In wild-type plants, this variation is mostly due to variation in seed phytic acid P, while the P_i and cellular P fractions of seed total P tend to remain constant (reviewed in Raboy, 1997).

Chemically induced, non-lethal recessive mutants that decrease seed phytic acid content have been isolated and genetically mapped in maize (*Zea mays* L.; Raboy and Gerbasi, 1996; Raboy et al., 2000) and barley (*Hordeum vulgare* L.; Larson et al., 1998; Rasmussen and Hatzak, 1998). These *low phytic acid* (*lpa*) mutations have the potential to alleviate the environmental and nutritional problems associated with phytic acid in animal feeds (Ertl et al., 1998). Low phytic acid crops may also offer improved nutrition for human populations that depend upon grains and legumes as staple foods. Moreover, these *lpa* mutants will provide a valuable system to study grain phytic acid synthesis.

Unlike the normal genetic and environmental affects that result in quantitative variation in seed total P, *lpa* mutants show large effects on the partitioning of P into phytic acid P, P_i, and lower Ins polyPs. The *lpa* mutations of maize and barley are characterized by two chromatographic phenotypes that are circumscribed by two respective genetic complementation groups, designated *lpa1* and *lpa2*, in each species. A wide range of phytic acid reductions are observed among different *lpa1* and *lpa2* mutants. However, all *lpa1* mutants are qualitatively identical in that phytic acid P reductions are counterbalanced by molar-equivalent increases in P_i. The *lpa1* loci have been mapped to maize chromosome 1S (centromere-distal; Raboy et al., 2000) and barley chromosome 2H (Larson et al., 1998). In contrast, the phytic acid reductions in *lpa2* mutants are matched by increases of P_i and lower Ins polyPs, in approximately equal parts. These *lpa2* loci have been mapped to maize chromosome 1S (centromere-proximal; Raboy et al., 2000) and barley chromosome 7H (Larson et al., 1998).

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Abbreviations: AFLP, amplified fragment length polymorphism; bp, base pair; HIP, high inorganic phosphorus; HPLC, high performance liquid chromatography; HVPE, high voltage paper electrophoresis; P_i, inorganic P; *lpa*, *low phytic acid*; Ins, *myo*-inositol; MIPS, *myo*-inositol 1-phosphate synthase; STS-PCR, sequence tagged site-polymerase chain reaction.

The synthesis of Ins P_6 can be summarized in two parts; the *early pathway* representing Ins and/or Ins P_1 synthesis and the *late pathway* representing Ins polyP metabolism. On the basis of the biochemical phenotypes of barley and maize *lpa* mutations, it has been hypothesized that *lpa1* mutations interrupt Ins or Ins P_1 synthesis and/or supply, whereas *lpa2* mutations interrupt Ins polyP metabolism (Larson and Raboy, 1999). The first committed step of Ins biosynthesis involves L-*myo*-inositol 1-phosphate synthase activity (MIPS, also referred to as D-*myo*-inositol 3-phosphate synthase), the only de novo source of the Ins ring in all organisms (Loewus, 1990). The gene for MIPS was first identified and cloned from yeast (Klig and Henry, 1984; Johnson and Henry, 1989) and subsequently from various higher plant species (Smart and Fleming, 1993; Johnson and Burk, 1995; Wang and Johnson, 1995; Ishitani et al., 1996). Yoshida et al. (1999) recently reported a single copy MIPS gene in rice (*Oryza sativa* L.) that shows temporal and spatial patterns of transcript accumulation corresponding with seed phytic acid synthesis.

As a first phase in the genetic study of phytic acid synthesis in cereal grains, we have isolated *lpa* mutants and compared their genetic map positions with those of MIPS loci. Larson and Raboy (1999) found that maize contains several dispersed MIPS loci, one of which maps to a site near and possibly identical to maize *lpa1* on chromosome 1S. Barley differs from maize in two ways (Larson and Raboy, 1999). First, the barley genome contains only one MIPS-homologous sequence located on a segment of chromosome 4 that is clearly orthologous to the maize chromosome 1S region containing a MIPS gene and *lpa1* mutation. Second, the barley *lpa1* mutation maps to a locus on chromosome 2H that is separate from the single copy MIPS gene on chromosome 4H. Here we report the isolation, inheritance and seed P phenotype of the first non-lethal rice *lpa1* mutant, and compare its map position to that of a single-copy rice MIPS gene developmentally upregulated at stages corresponding with seed phytic acid synthesis.

MATERIALS AND METHODS

Mutant Isolation

Gamma-irradiated seeds (M1) of five rice cultivars were planted and grown to produce the following generation of M2 seed. The M2 seeds produced by a given M1 plant were planted in a M2 row. Putative mutants are first referred to as their M2 row numbers. M3 seed was harvested, by panicles, from as many as 10 M2 plants in each row. Single panicles of M3 seed were sampled from five to 10 plants of each M2 row and tested for the *High Inorganic P* (HIP) phenotype typical of all the *lpa* mutants isolated to date. Individual M3 seeds were weighed, crushed, and extracted overnight in 10 μL 0.4 *M* HCl per mg (approximate seed wt.) at 4°C. Samples (10 μL) of these extracts were mixed with 90 distilled water and 100 μL of colorimetric reagent [1 volume 3 *M* H_2SO_4, 1 volume 2.5% (w/v) ammonium molybdate, 1 volume 10% (v/v) ascorbic acid (stored at 4°C), and 2 volumes distilled water] made fresh daily, as described by Chen et al. (1956). Assays were incubated at room temperature for approximately 1 h, so that individual seed extracts could be visually scored for the presence or absence of HIP. Each microtiter plate included five P standards made be appropriate dilutions of 1m*M* K_2HPO_4 to achieve (i) 0.0 μg P; (ii) 0.15 μg P; (ii) 0.46 μg P; (iv) 0.93 μg P; and (v) 1.39 μg P. In practice any seed extracts testing higher than the third P standard (0.46 μg P) were deemed HIP. If one or more seed displayed a HIP phenotype in the M2 progeny (M3 seed), the remnant extract samples were then tested for a corresponding reduction in phytic acid P by means of a high voltage paper electrophoresis (HVPE) assay for inorganic P (P_i) and Ins Ps (Raboy et al., 1990). M2s were selected for further study if a substantial reduction (≥20% as compared with wild type) in seed phytic acid P, corresponding to the observed increase in P_i, was detected with HVPE. Remnant M3 seed from these M2 mutants were planted, and the M4 and M5 generation seed was analyzed as above to identify heritable mutations.

Analyses of Seed P and Ins P Fractions

Samples of seeds were dried for 48 h at 60°C, milled to pass through a 2-mm screen, and stored in a desiccator until analysis. Seed total P was determined following wet-ashing of aliquots of tissue (typically 150 mg) and colorimetric assay of digest P (Chen et al., 1956). The ferric-precipitation method was used to determine phytic acid P (Raboy et al., 1990). Aliquots of tissue (typically 0.5–1.0 gm) were extracted in 0.4 *M* HCl:0.7 *M* Na_2SO_4. Phytic acid P was obtained as a ferric precipitate, wet-ashed and assayed for P as in the total P analysis. Seed P_i was determined colorimetrically following extraction of tissue samples (typically 0.5 g in non-mutant seeds and 0.15 g in mutant seeds) in 12.5% (w/v) TCA:25 m*M* $MgCl_2$. To facilitate comparisons, all seed P fractions are expressed as their P (atomic weight 31) content.

An HVPE assay for acid-extractable Ins Ps was performed as previously described (Raboy et al., 1990). Anion-exchange HPLC analyses of seed Ins Ps were performed by a modification of the methods as described (Phillippy and Bland, 1988; Rounds and Nielsen, 1993). Samples of seeds were dried, milled, and extracted in 0.4 *M* HCl overnight. Following centrifugation (10 000 *g*, 10 min), supernatants were filtered through Whatman No. 1 filter paper, and passed through Millipore HV 0.45-μm filters. Aliquots were then fractionated on a Dionex IonPac AS7 anion-exchange column, equipped with a Dionex IonPac AG7 guard column, which had been equilibrated with 10 m*M* methyl piperazine, pH 4.0 (Buffer A). The Ins phosphates were then eluted with the following gradient system at a flow rate of 0.5 mL min^{-1}: 0 to1 min 100% Buffer A; 1 to 26 min a concave gradient from 0 to 15% 1 *M* $NaNO_3$, pH 4.0 (Buffer B); 21 to 41 min a linear gradient from 15 to 100% Buffer B. The column elutent was mixed with colorimetric reagent [0.015% (w/v) $FeCl_3$:0.15% (w/v) sulfosalicylic acid) at a flow rate of 0.5 mL min^{-1}, and passed through a 290-cm reaction coil prior to peak detection via absorbance at 550 nm. Ins P in a sample peak was calculated by means of the following standard curve, obtained via the analysis of four Na Ins P_6 standards containing 24.9, 49.7, 74.6 and 99.5 n*M* Na Ins P_6; n*M* Ins P = 1.66×10^{-5} (Peak Area) − 3.85, $R^2 = 0.99$.

Inheritance and Mapping Studies

A rice *lpa1* mapping population was generated from an intersubspecific cross; a true-breeding 'Kaybonnet' *lpa1* mutant (*tropical japonica*) × wild-type 'ZHE733' (*indica*). Segregation of the *lpa1* trait was determined by progeny testing 137 F_2 plants by the HIP assay. Sixteen individual F_3 kernels, from each F_2 plant were assayed. The F_2 plants were classified as

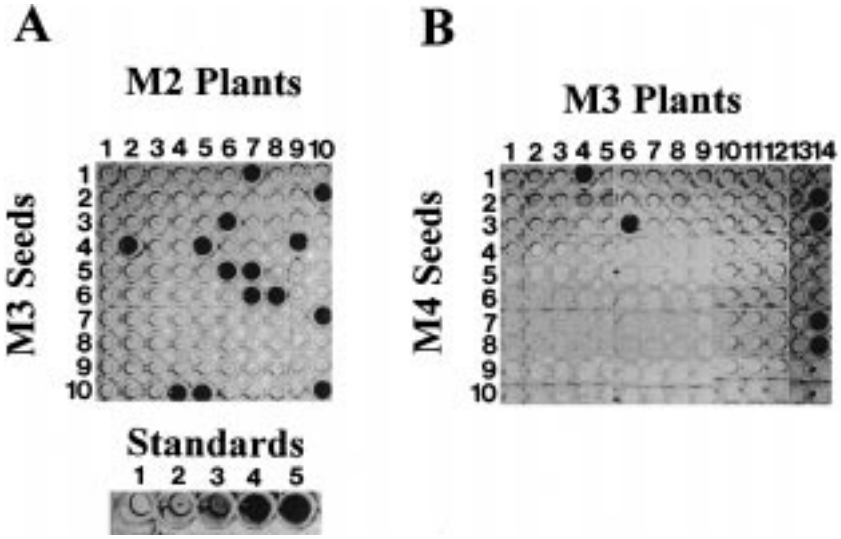

Fig. 1. Segregation of Orion M2-70 in the M2 and M3 generations. Seeds produced by a given plant were individually crushed and assayed for inorganic P. (A) M2 generation; 10 M3 seeds were tested for each of 10 Orion M2-70 M2 plants. (B) M3 generation; 10 M4 seeds were tested for each of 14 Orion M2-70 M3 plants. Standards: (1) 0.0 μg P; (2) 0.15 μg P; (3) 0.46 μg P; (4) 0.93 μg P; (5) 1.39 μg P. In this study, wild-type rice seeds typically have inorganic P levels (= 0.15 mg P_i g^{-1}) that yield an assay result < Standard No. 2. Orion M2-70 seeds have inorganic P levels resulting in an assay = Standard No. 5, indicating these seeds contained > 1.5 mg P_i g^{-1}.

homozygous wild type (+/+) if zero F_3 kernels were HIP, heterozygous (+/*lpa1*) if at least one F_3 progeny kernel was HIP, and homozygous mutant (*lpa1*/lpa1) if virtually all F_3 progeny kernels were HIP. Allowances were made for up to three phenotypically normal (+/*lpa1*) F_3 kernels identified from several *lpa1*/*lpa1* plants evidently contaminated by genetically dominant wild-type pollen, as determined by the distribution of HIP / wild-type F_3 ratios.

DNA samples of all homozygous +/+ or *lpa1*/*lpa1* F_2 plants were reconstructed from a bulk of 10 F_3 coleoptiles from each F_2 family. The AFLP analyses were performed as described by Vos et al. (1995). The AFLP marker loci designations described in this study correspond to the following *Eco*RI (E) and *Mse*I (M) AFLP primers with two and three selective nucleotides, respectively:

E13) GACTGCGTACCAATTCAG
M59) GATGAGTCCTGAGTAACTA
M61) GATGAGTCCTGAGTAACTG

The E13 primer was end labeled with T4 polynucleotide kinase and ATP[γ-^{33}P] before selective amplification. Selective amplification products were fractionated by 6% (w/v) denaturing PAGE (polyacrylamide gel electrophoresis) and visualized by autoradiography. A DNA size standard (10-bp ladder), end labeled with T4 polynucleotide kinase and ATP[γ-^{33}P], was included in one lane of each gel.

The F_2 intercross model of MAPMAKER version 3.0b (Lander et al., 1987) was used to identify AFLP markers genetically linked to the rice *lpa1* mutation. The F_2 backcross model of MAPMAKER was used to map genetically AFLP markers linked to rice *lpa1* in a population comprised of 135 IR64 (*indica*) and Azucena (*tropical japonica*) F_1 doubled haploids, characterized with 175 skeletal RFLP markers (Guiderdoni et al. 1992; Huang et al. 1994; and Huang et al. 1997; and kindly provided by B. Courtois, IRRI, Manila, The Philippines). This population was also used to map the rice MIPS gene as described below.

Microsatellite primers pairs were custom made for loci corresponding with the chromosomal map location of rice *lpa1*, based on sequences described by Panaud et al. (1996) and Chen et al. (1997), with 6-FAM 5′ conjugates on the forward primer sequences. Microsatellite amplification products and ROX-labeled internal lane size standards, were fractionated on 6% (w/v) denaturing PAGE gels and analyzed by mens of an automated, fluorescent DNA sequencer.

PCR primers (Saiki et al., 1985) for the rice MIPS gene were designed from the rice MIPS sequence reported by Yoshida et al. (1999) and selected from sites intended to amplify the same 3' regions corresponding to the MIPS STS-PCR restriction site polymorphisms mapped to barley chromosome 4 and maize chromosome 1S (Larson and Raboy, 1999). The sequence of these primers were as follows (F = plus strand, R = minus strand):

*Os*MIPS1083F) TGTGGTCGATGACATGGTCT
*Os*MIPS1529R) AGGATCATGTTGTTCTCGGG

These primers are referenced using numbers relative to the translation initiation codon of the barley, maize, and rice MIPS genes. STS-PCR analysis was performed as described by Larson and Raboy (1999), except that the final magnesium concentration was adjusted to 1 m*M* and the annealing temperature was 60°C.

RESULTS

Isolation and Seed P Phenotype of Rice *lpa1-1*

Preliminary analyses (data not shown) indicated that non-mutant rice cultivars grown under normal agronomic field conditions produced seed with 3 to 4 mg total P gm^{-1}, of which approximately 70% is found as phytic acid P and approximately 2% as P_i. Lower Ins P's if present were below the level of sensitivity of the chromatographic methods used here. Identical methodology routinely detects trace levels of Ins penta*kis*phosphates in maize and barley seeds. Thus phytic acid P represents ≥95% of acid-extractable Ins P in these wild-type rice seeds. M3 seed representing a total of 3632 M2 rows were screened for HIP, and putative mutants were observed to segregate in two M2 rows; Orion M2-

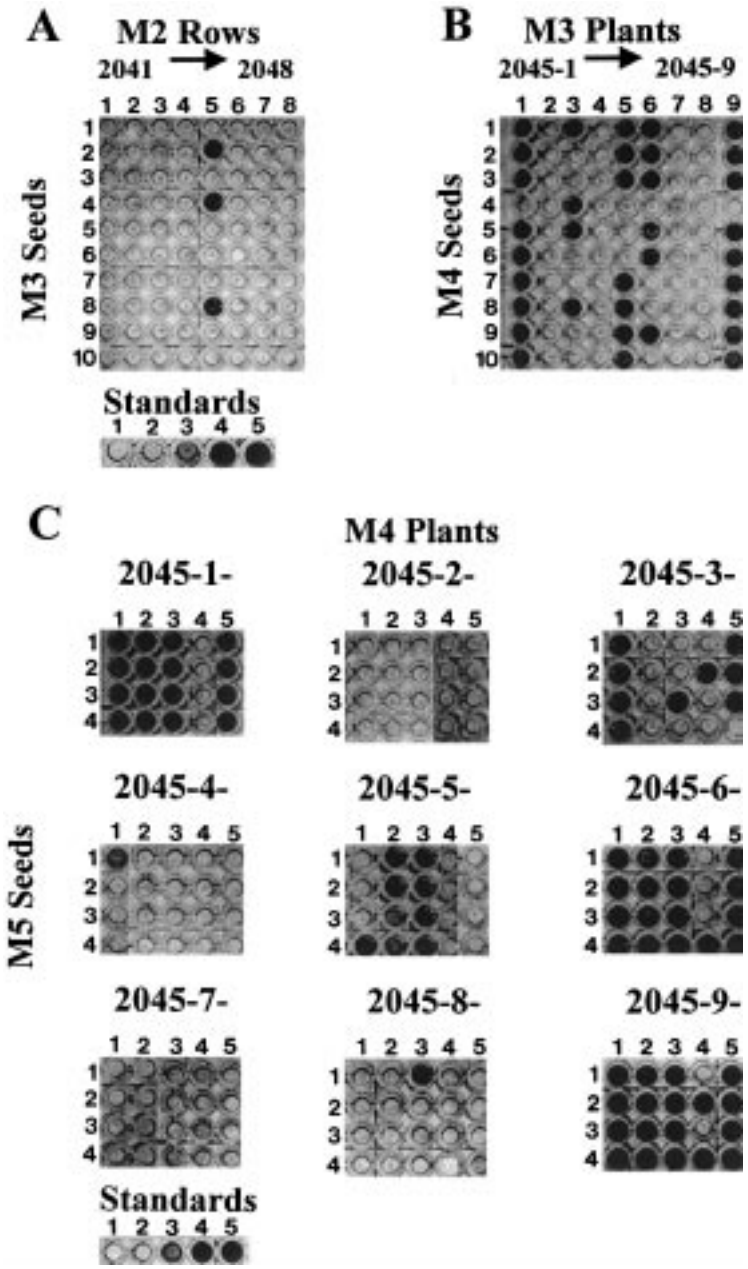

Fig. 2. Segregation of Kaybonnet M2-2045 in the M2 through M4 generations. Seeds produced by a given plant were individually crushed, and assayed for inorganic P. (A) M2 generation; A single M3 seed from each of 10 plants comprising a given M2 row were crushed, and tested. The rows shown are M2-2041 through M2-2048. (B) M3 generation, M3-2045-1 through M3-2045-9; 10 M4 seeds from each of nine M3 plants were tested. (C) M4 generation; a total of eight M5 seeds were tested for each of five sibling M4 plants representing the nine M3s in (B). The first four M5 tests for each M4 are shown. Standards: (1) 0.0 μg P; (2) 0.15 μg P; (3) 0.46 μg P; (4) 0.93 μg P; (5) 1.39 μg P. In this study, wild-type rice seeds typically have inorganic P levels (= 0.15 mg P_i g^{-1}) that yield an assay result < Standard No. 2. Kaybonnet M2-2045 seeds have inorganic P levels that yield an assay result typically ~ Standard No. 4, indicating these seeds contained ~ 1.0 mg P_i g^{-1}.

70 and Kaybonnet M2-2045. Initial assays of M3 seed produced by 10 Orion M2-70 M2 plants indicated that 8 of 10 plants produced one or more seed displaying a HIP phenotype, with an elevated P_i concentration representing about 50 to 75% of the seed total P concentration typical of non-mutant seed (Fig. 1A). However, the Orion M2-70 mutant was not obtained as a viable homozygote in the M3 generation (Fig. 1A) nor in the M4 (Fig. 1B) generation. Frequent sterility was observed in M3 plants, and the HIP phenotype was observed in tests of only three of 14 M3 plants. No further studies of this apparently lethal mutant have been conducted to date.

For initial screening of the Kaybonnet M2 population, single M3 seeds were sampled from panicles representing each plant in the M2 row. In the case of the M2-2045 row, three M3 seeds of nine tested displayed a HIP phenotype where the elevated P_i represented from 30 to 50% of non-mutant seed total P (Fig. 2A). Remnant M3 seed from nine M2 plants were planted, and tests of the M4 seed produced indicated recovery of the mutation in five of the nine M3s, with two apparently

Table 1. Seed dry weight and phosphorus (P) fractions[a] in wild-type (+/+) and homozygous *lpa1-1* genotypes (−/−). Values are means of duplicate analyses for each individual, on a dry weight basis.

Genotype	Plant	Seed dry weight	Total P	Phytic acid P	Inorganic P
		mg seed^{-1}		mg g^{-1} (% of Total P)	mg seed^{-1}
+/+	1	18.7	3.04	2.23 (73)	0.15 (5)
+/+	2	19.6	3.20	2.22 (69)	0.13 (4)
−/−	1	15.4	3.94	1.52 (39)	1.27 (32)
−/−	2	18.3	3.15	1.22 (39)	0.99 (31)
$(+/+)_{Av} - (-/-)_{Av}$		2.3	−0.43	0.86* (32**)	−0.99* (−27***)

homozygotes, M3-2045-1, and M3-2045-9 (Fig. 2B). Five M4 plants were produced for each M3, and for M3-2045-1 and M3-2045-9, four of five M4s were homozygous (Fig. 2C). This heritable and non-lethal rice mutation was termed *low phytic acid 1-1* (*lpa1-1*).

Quantitative analyses of seed produced by wild-type and *lpa1-1* M5 homozygotes indicated that homozygosity for *lpa1-1* had no discernable effect on seed total P (Table 1). Phytic acid P represented 39% of total P in *lpa1-1* seed, as compared with 71% of total P in wild-type seed. P_i represented 32% of total P in *lpa1-1* seed, as compared with 5% of total P in wild-type seed. No unusual accumulations of lower Ins Ps were observed in HVPE analyses (data not shown). Anion-exchange HPLC (Fig. 3) confirmed the level of reduction in phytic acid P observed in quantitative analyses of *lpa1-1* as compared with wild type, and confirmed that this reduction is not accompanied by increases in lower Ins Ps as observed in maize and barley *lpa2* mutants. These results indicate that in field grown plants under nominal cultural conditions, homozygosity for *lpa1-1* reduces seed phytic acid P by approximately 45%. This reduction in phytic acid P is accompanied by a molar-equivalent increase in P_i, such that the sum of phytic acid P and P_i is similar to that observed in wild type. This phenotype is similar to that of maize and barley *lpa1* mutants. For such mutants the ferric-precipitation assay for acid-soluble Ins P provides a quantitatively accurate measure of phytic acid P (Raboy et al., 2000). Little if any difference in dry weight of mutant versus wild-type seed was observed (Table 1).

Inheritance and Mapping of Rice *lpa1-1*

The F_3 progeny tests revealed 28 +/+ F_2 plants, 81 +/*lpa1-1* F_2 plants, and 28 *lpa1-1*/*lpa1-1* F_2 plants (Table 2). These frequencies fit the expected 1:2:1 genotypic ratio for a single gene following a heterozygous mating (chi-square value of 4.21 indicates non-significant deviation at the $P = 0.05$ level, 2 df). The 81 heterozygous families showed 331 F_3 HIP kernels from a total of 1296 F_3 kernels tested (Table 2), which closely fits the expected 3:1 ratio for a recessive allele at a single gene (chi-square value of 0.2 indicates non-significant deviation at the $P = 0.05$ level, 1df). The frequency among mutant homozygotes segregating field-planted progeny indicates that homozygosity for *lpa1-1* had little effect on germination or viability under nominal production conditions.

The rice *lpa1-1* mutation was initially mapped to chromosome 2 (Fig. 4) by AFLP analysis of 16 *lpa1-1*/*lpa1-1* and 16 +/+ F_2 genotypes from the *lpa1* mapping popula-

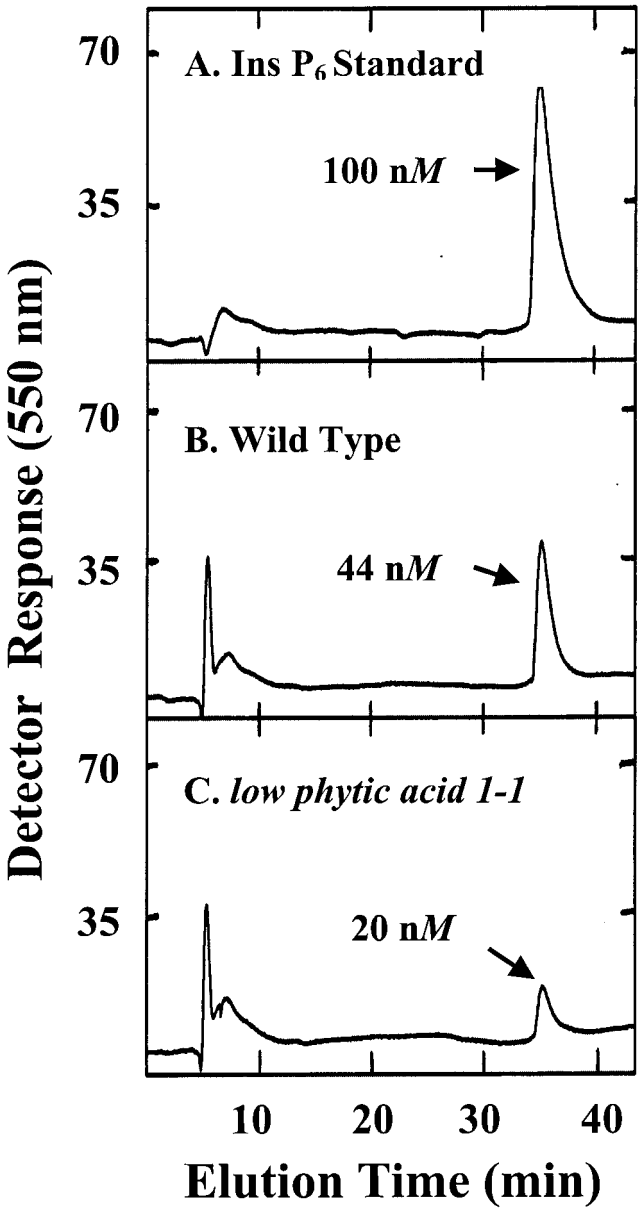

Fig. 3. Analysis of inositol phosphates in wild-type and *lpa1-1* rice seed. Standards or samples were loaded onto a strong-anion exchange column and eluted with a complex gradient. Ins Ps were detected following post-column derivitization via negative absorbance at 550 nm. (A) Elution of 100 nmol of *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate (Ins P_6) standard. (B, C) Equal amounts of wild-type (B) or *lpa1-1* (C) seed tissue were extracted, and equal aliquots of filtered supernatant were fractionated as in (A).

Table 2. Segregation of rice *lpa1-1* (−) and wild-type (+) alleles within and among 137 F_2:F_3 families derived from Kaybonnet (−/−) × ZHE733 (+/+).

F_3 Phenotypic ratio (HIP†/wild type)	Number of F_2 plants	Inferred F_2 genotype	Totals (HIP/wild type)
0/16	28	+/+	0/448
	28 = Total		0/448 = Total
1/16	8	+/−	8/128
2/16	8	+/−	16/128
3/16	14	+/−	42/224
4/16	21	+/−	84/336
5/16	11	+/−	55/176
6/16	11	+/−	66/176
7/16	4	+/−	28/64
8/16	4	+/−	32/512
	81 = Total		331/1296 = Total
13/16	1	−/−	13/16
14/16	3	−/−	42/48
15/16	2	−/−	30/32
16/16	22	−/−	352/352
	28 + Total		437/448

† F3 seeds produced by a given F2 plant were individually tested for the High Inorganic P phenotype (~1.0 mg P_i g^{-1}).

tion, 29 lines from the doubled haploid (DH) mapping population, and parents of these two mapping populations. Two AFLP primer pairs, E13M59 and E13M61, produced 22 polymorphisms common to the *lpa1* and DH mapping populations. Three of the latter polymorphisms were scored as codominant markers, evident by small length polymorphisms that appeared to be allelic in the DH mapping population. One of these codominant AFLPs was 26.1 cM (LOD = 5.2) from *lpa1-1* (in the *lpa1* mapping population) and mapped between the RZ58 and Amy1AC loci on rice chromosome 2 (in the DH mapping population). The codominant AFLP marker linked to *lpa1-1*, designated E13M61.286/7, was revealed as a 287-bp product in the *japonica* types (Kaybonnet *lpa1-1*/lpa1-1 and Azucena) and a 286-bp product in the *indica* types (ZHE733 and IR64). The remaining 28 AFLP markers showed no linkage to *lpa1-1* in the *lpa1* mapping population.

Five microsatellite markers, from the rice chromosome 2 region near Amy1AC, were analyzed in the rice *lpa1-1* mapping population (26 *lpa1-1*/*lpa-1* lines and 28 +/+ lines) to verify the map location of this mutation (Fig. 4). Three of these microsatellite markers were closely linked to the rice *lpa1-1* mutation. RM207, RM208, RM48 were 1.1 cM (LOD = 25.0), 1.1 cM (LOD = 24.4), and 2.2 cM (LOD = 23.2) from *lpa1-1*, respectively. The log-likelihoods of *lpa1-1*—RM207—RM208 and *lpa1-1*—RM208—RM207 are −1.78 and

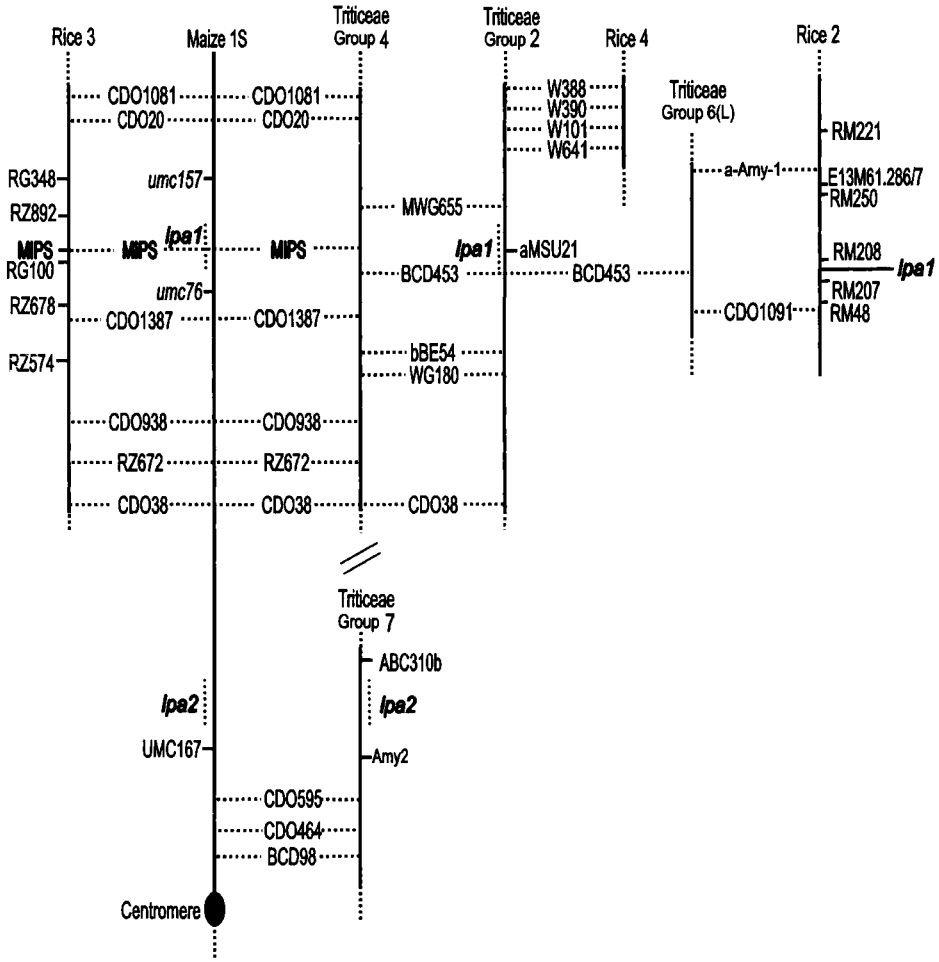

Fig. 4. Diagramatic map of chromosomal regions containing the *lpa1* and *lpa2* loci and myo-inositol 1-phosphate synthase (MIPS) genes of barley, maize, and rice. As determined in this study, the map locations of rice *lpa1-1* and MIPS gene are indicated on the rice chromosome 2 (outer right) and rice chromosome 3 (outer left) maps (Chen et al., 1997; Guiderdoni et al., 1992; Huang et al., 1994; Huang et al., 1997; and Panaud et al., 1997), respectively. Horizontal dashed lines indicate cross hybridization of RFLP probes, including MIPS (Ahn and Tanksley, 1993; Kurata et al., 1994; Larson and Raboy, 1999; VanDeynze et al., 1995) in addition to map locations of the maize and barley *lpa1* and *lpa2* mutations (Larson et al., 1998; Larson and Raboy, 1999).

1.80, respectively, relative to the most likely three point map order of RM207—*lpa1-1*—RM208. The overall order of RM48—RM207—RM208—RM250—RM221 was consistent between the DH mapping population (Chen et al., 1997) and *lpa1-1* mapping population (Fig. 4). With the order of these microsatellite markers fixed, the log-likelihoods of RM48—*lpa1-1*—RM207—RM208—RM250 and RM48—RM207—RM208—*lpa1-1*—RM221 are −2.16 and −3.25, respectively, relative to the most likely order of RM48—RM207—*lpa1-1*—RM208—RM250 as indicated in Fig. 4.

Map Position of Rice MIPS

The STS-PCR primers used in this study (*Os* MIPS1083F + *Os*MIPS1520R) specifically amplified a ~800-bp product (see Uncut portion of Fig. 5) from five genotypes of rice genomic DNA. This product is similar to STS-PCR sequences obtained from barley and maize (Larson and Raboy, 1999), but varies by length of three introns, at identical locations. The ~800-bp MIPS STS-PCR product of five rice genotypes was digested with nine, four-base cutter restriction enzymes that yielded a total of 25 fragments (results not shown). The ~310-bp *Scr*FI fragments observed in IR64 and ZHE733 (*indica* genotypes) were evidently cleaved at another *Scr*FI site to produce ~270-bp fragments in Azucena and Kaybonnet (*japonica* genotypes) (Fig. 5). Segregation analysis of this ~40-bp length polymorphism in the DH mapping population indicated that this MIPS marker maps to a locus on rice chromosome 3 that is evidently orthologous to the MIPS loci on maize chromosome 1S and Triticeae 4H (Fig. 4). By two-point map analysis, this MIPS gene was 7 cM (LOD = 18.1) from the RG100 locus. Multi-locus map analysis of marker in this region indicated scoring errors in more than one of these loci and that MIPS may actually be within 3 cM of RG100. Nevertheless, the general order of markers in our analysis (Fig. 4) is congruent with previously reported maps for this DH mapping population (Guiderdoni et al., 1992; Huang et al., 1994, 1997) and other rice mapping studies.

DISCUSSION

Mutations that reduce seed phytic acid have now been isolated, characterized, and genetically mapped in three major cereal crop species: barley, maize, and rice. This comparative approach will contribute to a robust understanding of seed phytic acid synthesis and cereal quality genetics. The viability and near wild-type mature seed dry weight of homozygous *lpa1-1* rice mutants provides evidence that wild-type phytic acid levels are not essential to seed function. These findings are consistent with previous studies of maize and barley *lpa* mutants, which challenge the widely held view that phytic acid is an essential sink used to maintain cellular P homeostasis during seed development. An alternate explanation is that phytate salts, containing much of the mineral nutrients stored in many plant seeds (Lott et al., 1995), are synthesized and stored as a mechanism to prevent leaching of P and other mineral nutrients from the natural seed bank. However, this putative role is probably not required in domesticated crops. Agronomic evaluations of different cereal genotypes bred with *lpa* mutations, such as rice *lpa1-1*, will provide an empirical test of this hypothesis.

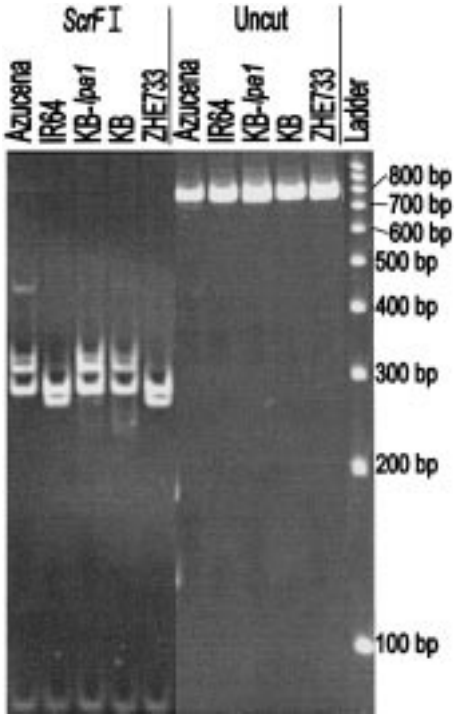

Fig. 5. A rice *myo*-inositol 1-phosphate synthase (MIPS) STS-PCR restriction polymorphism analyzed by 6% (w/v) PAGE. This polymorphism was mapped to rice chromosome 3 in the IR64 × Azucena doubled haploid mapping population.

Inheritance studies of *lpa1* mutations indicate that they are recessive and that translocation of gene products or metabolites from the maternal plant to the developing seed does not appear to complement the loss of function in homozygous, *lpa1-1*/*lpa1-1* seeds produced by heterozygous, +/*lpa1-1* maternal rice, barley (Larson et al., 1998), or maize (Raboy et al., 2000) plants. Therefore the phytic acid reductions in *lpa1* seeds are a result of metabolic changes that affect the developing seed per se. Sakri and Shannon (1975) demonstrated that [2-^{3}H]*myo*-inositol, injected into developing wheat spikes, was translocated to the kernels where it appeared in the phytic acid-rich bran fraction. This observation suggests that seed-specific MIPS activity may not be the only source of Ins available for phytic acid synthesis. However, Yoshida et al. (1999) recently reported a single copy MIPS gene in rice that shows temporal and spatial patterns of elevated transcript accumulation corresponding with phytic acid synthesis during seed development. The mapping experiments described in this study demonstrated that this MIPS gene maps to a locus on rice chromosome 3, which is orthologous to the MIPS gene near maize *lpa1* on chromosome 1S and the barley MIPS gene on Triticeae chromosome 4. Homozygosity for several maize *lpa1* mutations, which map near the canonical MIPS gene on maize chromosome 1S, result in a near absence of seed phytic acid (unpublished data, 1995). Therefore, expression studies of this canonical MIPS gene in rice (Yoshida et al., 1999) and genetic map location near maize *lpa1* suggest that this MIPS

gene may be an appropriate target for seed-specific control of Ins polyphosphate metabolism.

Since rice *lpa1* and barley *lpa1* loci are not coincident with single-copy, canonical MIPS genes identified in these species, they may represent additional, non-canonical MIPS targets of potential value in manipulating seed phytic acid content. It is possible that barley *lpa1* and/or rice *lpa1-1* are mutations of cryptic MIPS genes; sequences encoding a protein with MIPS activity but having little or no homology to the canonical MIPS sequence . Another possibility is that these barley and rice *lpa1* mutations affect trans-acting regulatory factors that control expression or activity of MIPS.

MIPS regulatory loci have not yet been identified in higher plant species; however, at least 10 different Ins auxotrophy genes have been identified in yeast (Culbertson and Henry, 1975), albeit several of these have pleiotropic effects on carbon source utilization. The majority of yeast auxotroph mutants map to the *ino1* locus, the MIPS structural gene (Donahue and Henry, 1981); however, two other loci, *ino2* and *ino4*, also show more than one allelic representative. Studies suggest that gene products of *ino2* and *ino4* interact as transcription factors of MIPS (Ambroziak and Henry, 1994; Hoshizaki et al., 1990). A search of Genbank failed to reveal any rice sequences homologous to the yeast *ino2* or *ino4* nucleotide sequences (results not shown). Another possibility is that barley *lpa1* and/or rice *lpa1* may be structural loci encoding one of at least two other functions believed to play a role in Ins synthesis and supply: Ins 1-phosphatase or Ins 1-kinase. On the basis of the low level of Ins polyphosphates other than phytic acid observed in *lpa1* mutants, we still believe the effect of these mutations precedes Ins phosphorylation pathways.

The issue of dietary phytic acid in human nutrition and health is complex. Dietary phytic acid may have positive roles as an anti-oxidant or anti-cancer agent, or negative roles as a contributor to mineral deficiency (Harland and Morris, 1995). The impact or role of phytic acid in human nutrition may vary as a result of dietary differences within and among populations. For example, phytic acid's negative roles may outweigh any positive roles for growing children and child-bearing women in developing countries that depend on whole grains foods for the bulk of nutritional calories. The potential value of a low phytate rice is further complicated by the fact most grain phytic acid is localized in the aleurone and scutellum (O'Dell et al., 1972; Yoshida et al., 1999). Since rice is primarily consumed as a milled product, most grain phytic acid is removed in the bran fraction. Thus, in terms of nutritional value, milled rice produced from an *lpa1-1* line may not differ greatly from milled rice from a wild-type line. Since phytic acid represents the major sink for P and mineral deposition in the aleurone layer, studies are underway to determine if in addition to altering the chemistry of seed P, mutations like rice *lpa1-1* alter the distribution of P and nutritionally important minerals like iron and zinc in the seed. Any increases in the level of these nutrients in the endosperm fraction (i.e., milled rice) would represent an improvement in nutritional value. In any case, the bran fraction of rice *lpa* genotypes would be of greater nutritional value when used in animal feeds, and reduce the environmental problems associated with manure phosphates from non-ruminant livestock.

Mutants like rice *lpa1-1* are currently being used to breed first generation low phytate crops for use in foods and feeds, although the agronomic acceptability of *lpa* genotypes has yet to be determined. Regardless of whether or not *lpa* genotypes are widely adopted, isogenic lines with and without these *lpa* mutations will enable new experimental approaches to examine the nutritional merits of phytic acid in human health and livestock production.

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